Glutaraldehyde: Nature of the Reagent

A. H. Korn, S. H. Feairheller and E. M. Filachione

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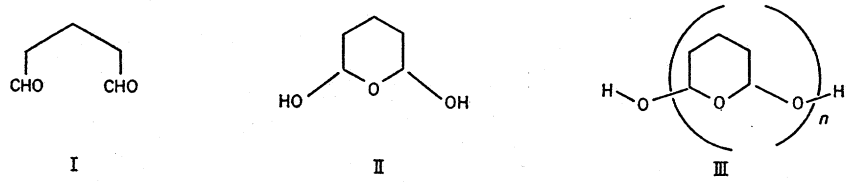
Aqueous solutions of glutaraldehyde used for the chemical modification and stabilization of proteins have been found to consist of free glutaraldehyde (I),



I II III

the cyclic hemiacetal of its hydrate (II) and oligomers of this (III) in equilibrium with each other. Ultraviolet absorption spectra of these solutions at wavelengths greater than 200 nm should have only a single maximum at 280 nm. Absorption at 235 nm is due to an impurity which can be removed by various means. Reactions of the reagent with proteins involve principally the lysinyl (and hydroxylysinyl) residues in the relative amounts of four moles of glutaraldehyde to one of lysine. Three unstable products can be partially isolated from acid hydrolyzates of glutaraldehyde-treated proteins or from the reaction mixtures of glutaraldehyde and model compounds; two of these products have strong ultraviolet absorption near 265 nm.

A group of scientists at this laboratory has been working on a study of the reaction of glutaraldehyde (pentanedial) with proteins (mainly collagen) for a number of years. Because of our interest (Fein, Harris, Naghski & Filachione, 1959; Filachione, Korn & Ard, 1967; Korn & Filachione, 1967) in the reagent as well as the interest of others in its more general use as a protein cross-linking reagent (Bishop & Richards, 1968; Bowes & Cater, 1966; Habeeb & Hiramoto, 1968; Munton & Russell, 1970; Roux & Hillman, 1969; Schejter & Bar-Eli, 1970; Wang & Tu, 1969) we wish to report the results of a study we have made on the nature of the reagent itself in aqueous solution and, in particular, to correct some inaccuracies that have appeared in the literature concerning the reagent and its reaction with proteins. This study consisted of a detailed investigation of several physical and chemical properties of six commercial samples of glutaraldehyde. These six samples are identified by letters and are as follows: A, 70% glutaraldehyde solution sealed in glass ampoules under an inert gas; B, 50% glutaraldehyde solution containing some methanol; C, same as B but from a different source; D, 25% glutaraldehyde solution containing some methanol; E, same as D but containing more methanol which is indicated on the label; and F, same as D but an old sample which was discolored and had a solid residue on the bottom of the bottle. Samples A to E had been purchased just before this study; however, aging over a period of one year caused no changes in the chemical or physical properties of sample A. The others, B to F, were not studied over this extended period of time.

Five of the six samples, A through E, had an absorption maximum at 280 nm and the sixth sample, F, had a shoulder at this wavelength. For two samples, A and C,

this was the only maximum above 200 nm and the extinction coefficient was 3·6. In addition to this maximum, the other samples exhibited a maximum at about 235 nm. For sample F, run at a higher dilution, this latter was the only maximum.

We agree with the proposal (Hardy, Nicholls & Rydon, 1969) that the ultraviolet absorption maximum at 235 nm of certain commercial samples of glutaraldehyde is due to the presence of a very small amount of an impurity perhaps even of the type suggested by others (Richards & Knowles, 1968). However, even in the sample with the strongest absorption at 235 nm this cannot account for more than 1% of the glutaraldehyde. Glutaraldehyde itself in sample A and in other samples after purification by distillation (Hardy *et al.*, 1969) or by treatment with activated charcoal (Anderson, 1967) exhibits no absorption at this wavelength. Its only absorption maximum above 200 nm is the weak one at 280 nm.

The nuclear magnetic resonance spectra were measured on the samples as they were obtained in aqueous solution even though the water absorption obscured a part of the spectra. There were two reasons for doing this. First, it was our desire to examine the reagent without any alteration to it and, second, it was felt that changing the solvent to deuterium oxide might not only cause an exchange of deuterium for hydrogen bound to oxygen but also for hydrogen bound to carbon in the α-positions of glutaraldehyde. The spectra of all six samples were the same with the exception of absorption at 3·4δ (measured from tetramethyl silane (0·0δ) as an external standard). This absorption varied in intensity from sample to sample and was completely missing from sample A. The other absorption peaks with their relative intensities in brackets are as follows: broad absorption centered at 1·68δ with a partially separated multiplet on the downfield side at 1·90δ [18]; a triplet exhibiting further fine splitting centered at 2·60δ [2]; broad absorption at 4·77δ with a partially separated multiplet on the downfield side at 5·07δ [16]; an undefined multiplet at 5·31δ [3]; and a finely split triplet at 9·62δ [1].

TABLE 1

Effect of temperature on nuclear magnetic resonance absorption of glutaraldehyde solutions

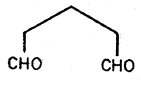
Temperature (°C)	Intensities of n.m.r. absorption maxima relative to aldehyde protons (9·62δ)						
	1·68δ	*1·90δ*	*2·60δ*	*4–5δ*	*5·07δ*	*5·31δ*	*9·62δ*
25	18		2	16		3	1
50	7		2	8		1	1
75	3		2	3	0·4	0·25	1
90	1	1	2	2	0·2	0·1	1

The effect that temperature had on the composition of sample A as determined by n.m.r. spectroscopy is summarized in Table 1. The only peak experiencing a change in chemical shift as the temperature was raised was the broad absorption at 4·77δ at room temperature. As the temperature was raised it shifted upfield to 4·56δ at 50°C, 4·25δ at 75°C and 4·03δ at 90°C. While the chemical shifts of the other peaks remained constant, their relative intensities did not. Finally, at 90°C, these changes revealed the entire spectrum with the following characteristics: broad absorption at 1·68δ; multiplet of multiplets centered at 1·90δ; triplet with further fine splitting at 2·60δ;

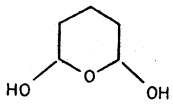
strong singlet at 4·03δ; broad triplet at 5·07δ; broad triplet at 5·31δ; and a finely split triplet at 9·62δ.

Acetylation of a lyophilized sample in pyridine solution gave a product which was isolated by chromatography and which had an n.m.r. spectrum with the following characteristics (again, relative intensities are in brackets): broad absorption at 1·73δ [6]; sharp singlet at 2·05δ which may have been composed of more than one peak [3]; and two broad multiplets at 5·2δ and 6·0δ [total relative intensity 2].

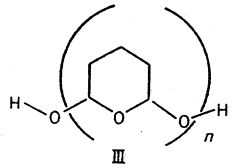
The n.m.r. data reported here agree well with those reported elsewhere (Richards & Knowles, 1968; Hardy *et al.*, 1969). The n.m.r. absorption at 3·4δ is undoubtedly due to the methanol present as has been suggested (Hardy *et al.*, 1969) and not a deshielded methylene group (Richards & Knowles, 1968). The other n.m.r. absorption maxima of the aqueous solutions can only be accounted for by the presence of the species I, II and III in these solutions. The strong absorption at 4·77δ at room temperature which experiences a change in chemical shift as the temperature is changed is that of the protons attached to oxygen (of the water and hydroxyl groups of II and III).



I



II

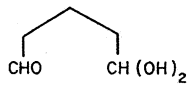


III

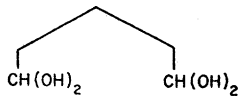
Calculations based on the several spectra run on sample A indicated that this sample contained about 32% water which agreed well with its stated composition (70% glutaraldehyde). The absorption maxima centered at 9·62δ and 2·60δ and the partially separated maxima centered at 1·90δ (at 25°C) can be assigned to the aldehyde, α-methylene and central methylene protons, respectively, of glutaraldehyde (I). The relative intensities agree quite well with these assignments. The maxima at 1·68δ and those at 5·07δ and 5·31δ can be assigned to the methylene protons and the acetal protons respectively of II and III. The shift of the water and hydroxyl proton peak upfield revealed the absorption at 5·07δ as a separate peak. Both it and the peak at 5·31δ are attributed to the acetol protons. It is not surprising that these protons absorb in two separate places because of the several different chemical species involved and the possibilities of geometrical isomerism. Again, the relative intensities agree.

The relative amounts of these three species in sample A change as the temperature is changed as is indicated by the n.m.r. spectra at different temperatures (Table 1). Calculations based on the total number of protons of each species from these n.m.r. data and the above assignments indicate the following relative amounts of I and II plus III (it does not distinguish between these latter two) at the various temperatures: 25°C, 15% I, 85% II and III; 50°C, 33% I, 67% II and III; 75°C, 60% I, 40% II and III; and 90°C, 75% I, 25% II and III.

The proposal (Hardy *et al.*, 1969) that these aqueous solutions consist only or mainly of II and the other two hydrates IV and V with little or no free glutaraldehyde is untenable in view of our results at elevated temperatures. Our data can be reconciled with the presence of II, IV and V only at 25°C. At 50°C, only II and IV could be present while at 75 and 90°C, neither IV nor V can be present. The absorption in



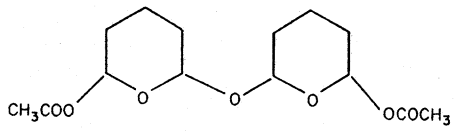
IV



V

the amounts that the presence of these species (IV and V) would require is simply missing from the spectra measured on the sample above room temperature. While some argument might be made that IV and V are present at lower temperatures but disappear at the higher temperatures, we feel that the structure of the spectra indicates the presence of free glutaraldehyde at all temperatures investigated and can be explained by the presence of just I, II and III.

The spectral data for the acetylated product also suggest the presence of species with the structures of III. In particular, this spectrum can best be explained by VI. The chemical shifts and relative intensities both agree.



VI

While only of a qualitative nature, one observation which has been made several times indicates the presence of a water-insoluble material in the concentrated (70%) glutaraldehyde solutions. When these are diluted by being poured into water, there is an initial cloudiness which rapidly disappears on mixing. This agrees well with our conclusion that III is present in equilibrium with I and II and is converted rapidly to I and II on dilution. The number average molecular weight determinations on sample A also support this conclusion. A value of 215 indicates the presence of some oligomers with n greater than 2.

All of these samples react as free glutaraldehyde under acidic and neutral conditions of pH. The yields of the 2,4-dinitrophenylhydrazone derivative were at least quantitative. A 131% yield for sample E indicates that the concentration stated on the label must be incorrect since this reaction appears to be a reliable measure of the amount of reagent present in the other five samples. Also, it has been demonstrated in the papers already cited (Fein *et al.*, 1959; Korn & Filachione, 1967) that there is a direct correlation between the amount of unreacted glutaraldehyde in solution (as determined iodometrically) and the amount that has combined with the protein (as determined by the absorption at 265 nm of hydrolyzates).

There was a rather consistent relationship between the amount of glutaraldehyde combined with the protein and the amount of lysine plus hydroxylysine consumed. This was close to 4 moles of glutaraldehyde per mole of lysine plus hydroxylysine. This same relationship has been found for wool (Happich, Taylor & Feairheller, 1970).

It has been established by using model compounds (N_α-carbobenzoxy-L-lysine, hippuryl-L-lysine and poly-L-lysine) that the development of the absorption at 265 nm

is associated with reactions of the lysinyl residues in proteins and not with reactions of tyrosyl residues as has been suggested (Habeeb & Hiramoto, 1968). It is unfortunate that these workers chose a peptide that contained neither tyrosine nor lysine to test their hypothesis. Attempts have been made to identify the products responsible for the absorption but, due to their apparent instability, this has been only partially successful. There are apparently three products formed, two with ultraviolet absorption and one without. The chromophoric group that is responsible for this absorption has been found to be sensitive to reducing agents and is destroyed by sodium borohydride. It is apparently insensitive to mild oxidizing agents.

These aqueous solutions of glutaraldehyde are not as complicated as indicated previously (Richards & Knowles, 1968) and most commercial samples are essentially the same with the exception of the methanol. The purity can be checked by ultraviolet analysis of the solutions. The reactive species present in these solutions is apparently the free glutaraldehyde and not a condensation polymer as has been suggested (Richards & Knowles, 1968). The reaction of the reagent with proteins appears to involve mainly lysinyl residues but is complicated in nature yielding a mixture of at least three products. The extent of the reaction can be determined by the iodometric analysis of the solution or the empirical ultraviolet analysis (Korn & Filachione, 1967) of hydrolyzates if the amount of aromatic amino acids is low. It should be pointed out that under alkaline conditions, glutaraldehyde does polymerize in a different, less well understood fashion (Hardy *et al.*, 1969) and that under these conditions does not react well with proteins (Bowes & Cater, 1966).

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